Arsenite-Mediated Promotion of Anchorage-Independent Growth of HaCaT Cells through Placental Growth Factor

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Various cancers including skin cancer are increasing in 45 million people exposed to arsenic above the World Health Organization's guideline value of $10\,\mu g\,l^{-1}$. However, there is limited information on key molecules regulating arsenic-mediated carcinogenesis. Our fieldwork in Bangladesh demonstrated that levels of placental growth factor (PlGF) in urine samples from residents of cancer-prone areas with arsenic-polluted drinking water were higher than those in urine samples from residents of an area that was not polluted with arsenic. Our experimental study in human nontumorigenic HaCaT skin keratinocytes showed that arsenite promoted anchorage-independent growth with increased expression and secretion of PlGF, a ligand of vascular endothelial growth factor receptor1 (VEGFR1), and increased VEGFR1/mitogen-activated protein kinase/ERK kinase (MEK)/extracellular signal–regulated kinase (ERK) activities. The arsenite-mediated promotion of anchorage-independent growth was strongly inhibited by PlGF depletion with decreased activities of the PlGF/VEGFR1/MEK/ERK pathway. Moreover, arsenite proteasome-dependently degrades metal-regulatory transcription factor-1 (MTF-1) protein, resulting in a decreased amount of MTF-1 protein binding to the *PlGF* promoter. MTF-1 negatively controlled *PlGF* transcription in HaCaT cells, resulting in increased *PlGF* transcription. These results suggest that arsenite-mediated MTF-1 degradation enhances the activity of PlGF/VEGFR1/MEK/ERK signaling, resulting in promotion of the malignant transformation of keratinocytes. Thus, this study proposed a molecular mechanism for arsenite-mediated development of skin cancer.

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INTRODUCTION

Inorganic arsenic, a potential carcinogen in humans, is a common and widespread environmental contaminant for drinking well water (National-Research-Council, 1999; IARC, 2004). In fact, tens of millions of patients with arsenicosis have been reported in residents who drink arsenic-polluted well drinking water in the world (National-Research-Council, 1999; IARC, 2004). Previous epidemiological studies showed that various cancers are increasing in patients with arsenicosis (IARC, 1980, 2004; Smith *et al.*, 1992; Kato *et al.*, 2010; Yajima *et al.*, 2012). Cutaneous squamous cell carcinoma is one of the representative cancers induced by arsenic (Lansdown, 1995; IARC, 2004). However, there is limited information on the molecular mechanism of arsenic-mediated cancer despite its worldwide prevalence. Clinically available molecular targets for prediction, prevention, and therapy for arsenic-mediated cancer have not been sufficiently established.

The HaCaT keratinocyte cell line was established from human skin and a naturally immortalized cell line (Boukamp *et al.*, 1988). Morphogenesis and differentiation of HaCaT cells are similar to those of normal keratinocyte cells *in vitro* (Boukamp *et al.*, 1988). HaCaT cells are often used to analyze mechanisms for malignant transformation of keratinocytes induced by environmental factors such as ultraviolet light and heavy metals including arsenic (Kumasaka *et al.*, 2012; Yajima *et al.*, 2012). On the other hand, the A431 cell line derived from human squamous cell carcinoma is a representative malignant keratinocytic tumor cell line (Han *et al.*, 2010; Yajima *et al.*, 2012). HaCaT and A431 cells are concurrently used to compare the characteristics of nontumorigenic and tumorigenic cutaneous cells *in vitro* (Han *et al.*, 2010; Thang *et al.*, 2011; Yajima *et al.*, 2012).

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Abbreviations: ERK, extracellular signal–regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; mRNA, messenger RNA; MTF-1, metal-regulatory transcription factor-1; PlGF, placental growth factor; VEGF, vascular endothelial growth factor; VEGFR1, VEGF receptor 1

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Previous studies have shown that mitogen-activated protein kinase/ERK kinase (MEK)/extracellular signal–regulated kinase (ERK) activation has a central role in arsenic-mediated malignant transformation of keratinocytes (Kumasaka *et al.*, 2012).

Placental growth factor (PlGF) is a member of the vascular endothelial growth factor (VEGF) family and a ligand of VEGF receptor 1 (VEGFR1; Fischer *et al.*, 2007; Loges *et al.*, 2009). Interaction of PlGF and VEGFR1 has been reported to stimulate proliferation and invasion of tumor cells (Fischer *et al.*, 2007; Loges *et al.*, 2009). PlGF messenger RNA (mRNA) and protein levels were increased in several types of cancers including squamous cell carcinoma (Parr *et al.*, 2005; Cheng *et al.*, 2010). A phase I study using a mAb against PlGF for SCC has already started (Martinsson-Niskanen *et al.*, 2011; Lassen *et al.*, 2012). Previous studies revealed that several transcription factors directly bind to the promoter of the *PlGF* gene and regulate *PlGF* expression (Cramer *et al.*, 2005; Nishimoto *et al.*, 2009). These results suggest that PlGF has an important role in SCC.

Arsenic exposure through drinking well water increases the arsenic concentration of urine in humans (Chowdhury *et al.*, 2000). To our knowledge, however, there is no information on the relationships between urinary arsenic levels and urinary PlGF levels, between urinary PlGF levels and SCC development, and between PlGF function and arsenic-mediated cancer.

In this study, we investigated the molecular mechanism for arsenite-mediated anchorage-independent growth, a distinct phenotype of cancer formation, focusing on the regulation of PlGF expression via metal-regulatory transcription factor 1 (MTF-1) in HaCaT cells based on our fieldwork in residents of cancer-prone areas in Bangladesh.

RESULTS

Concentrations of urinary arsenic and PlGF in residents of Bangladesh

To elucidate the relationship between arsenic exposure and PlGF in humans *in vivo*, we performed fieldwork studies in cancer-prone areas (Samta and Tangra, Jessore; Kurokawa *et al.*, 2001; Yokota *et al.*, 2002; Tareq *et al.*, 2003) with arsenic-polluted well drinking water (mean ± SD $= 261.6 \pm 114.0\,\mu g\,l^{-1}$; $n = 15$) and an unpolluted area (Pullerhat, Jessore) with well drinking water that was not polluted with arsenic (mean ± SD $= 2.8 \pm 5.9\,\mu g\,l^{-1}$; $n = 10$) in Bangladesh. Mean concentrations of urinary arsenic and PlGF in residents of Samta and Tangra were $482.6\,\mu g\,g^{-1}$ creatinine and $33.2\,pg\,g^{-1}$ creatinine, respectively, whereas those in residents of Pullerhat were $160.4\,\mu g\,g^{-1}$ creatinine and $17.7\,pg\,g^{-1}$ creatinine, respectively (Figure 1). Thus, urinary arsenic and PlGF concentrations in residents of cancer-prone areas were about 3- and 2-fold higher, respectively, than those in residents of an arsenic-unpolluted area. As exposure of mice to arsenic through drinking water affected expression patterns and levels of proteins in the skin (Li *et al.*, 2011), we also treated *hairless* homozygous mice (*Hr/Hr*) with 30 μM arsenite through drinking water for 2 months to analyze the expression levels of PlGF protein in mouse skin

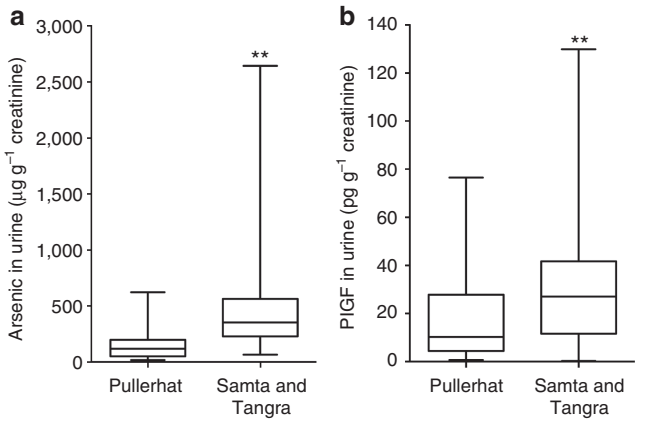

Figure 1. Levels of arsenic and PlGF in human urine samples in Bangladesh. Concentrations (box plot) of arsenic (**a**) and PlGF (**b**) in urine samples from residents of Samta and Tangra ($n = 61$) and Pullerhat ($n = 40$), Jessore in Bangladesh are presented. The boxes contain 50% of all values (observations between the 25th and 75th percentiles). The horizontal lines inside the boxes represent the medians. The bars extend from the boxes to the highest and lowest values. Data of urinary arsenic and PlGF were provided as creatinine-adjusted urinary arsenic and PlGF levels. Significantly different (**$P < 0.01$) from concentrations of arsenic or PlGF in urine from residents of Pullerhat by the Mann–Whitney *U*-test.

(Supplementary Figure S1 online). Immunohistochemical analysis showed that PlGF protein expression level in the epithelium of arsenite-exposed mice was 2.9-fold higher than that in control mice (Supplementary Figure S1c online). These results suggest that arsenite exposure through drinking water increased PlGF expression level in the epithelium mainly composed of keratinocytes. These results also suggest that an increase of PlGF level may be associated with arsenite exposure in humans and mice.

Arsenite-mediated promotion of anchorage-independent growth

To reveal the relationship between arsenite exposure and increase of PlGF level, we further examined the effect of PlGF on arsenite-mediated development of cancer *in vitro*. In previous studies, arsenic was detected in 80% of tissues of arsenic-related keratosis and Bowen's disease, and the maximum concentration was $61\,800\,\mu g\,kg^{-1}$ (824 μM; Matsui *et al.*, 1999). Previous studies have also shown that ratios of trivalent arsenite (As(III)) and pentavalent arsenate (As(V)) in human urine were 13.4% and 1.5%, respectively (Hata *et al.*, 2012). Furthermore, arsenic concentrations in nail samples (around $100{,}000\,\mu g\,kg^{-1}$) and hair samples ($10{,}000\,\mu g\,kg^{-1}$) from residents of the arsenic-polluted areas were 10- to 100-fold higher than those in their urine (Hinwood *et al.*, 2003; Gault *et al.*, 2008). Results of these previous studies suggest that cutaneous cells in residents of arsenic-polluted areas could be exposed to high concentrations of arsenite (As(III)). On the other hand, more than 10 μM arsenite (As(III)) was shown to be highly toxic for human nontumorigenic normal keratinocytes (HaCaT cells) (Boukamp *et al.*, 1988) in our *in vitro* studies, as previously shown in various kinds of cells (Yancy *et al.*, 2005). Therefore, 1, 3, and 10 μM concentrations

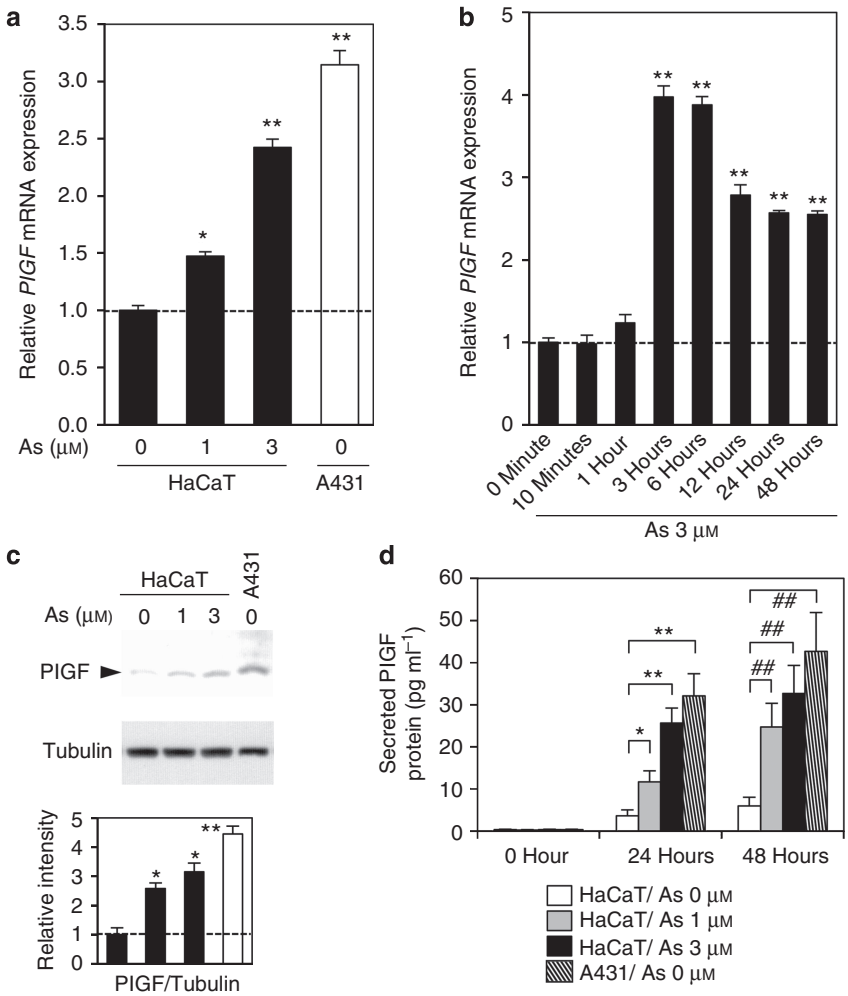

Figure 2. Arsenite-mediated expression of placental growth factor (*PlGF*) messenger RNA (mRNA) and protein. (**a**) Expression levels of *PlGF* mRNA evaluated by real-time PCR in HaCaT cells untreated or treated with 1 and 3 μM arsenite for 48 h (black bars) and in untreated A431 cells (a white bar) are presented. The results are presented as ratios (mean ± SD; $n = 3$) of *PlGF* mRNA relative to untreated HaCaT cells. (**b**) The time course of *PlGF* mRNA expression in HaCaT cells treated with 3 μM arsenite is presented. The results are presented as ratios (mean ± SD; $n = 3$) of *PlGF* mRNA relative to untreated control HaCaT cells. (**c**) Expression levels of PlGF protein evaluated by immunoblotting in HaCaT cells treated with 1 and 3 μM arsenite for 48 h and in untreated A431 cells. Intensities of bands are presented as ratios (mean ± SD; $n = 3$) relative to untreated HaCaT cells. Amounts of tubulin are also presented as an internal control. (**d**) Amount of secreted PlGF protein in the culture medium evaluated by ELISA is presented. Significantly different from untreated HaCaT cells (*$P < 0.05$; **$P < 0.01$ in **a**–**c**), from untreated HaCaT cells for 24 h (*$P < 0.05$; **$P < 0.01$ in **d**) or 48 h ($^{\#\#}P < 0.01$ in **d**) by Student's *t*-test.

of arsenite were used in our *in vitro* study. Although 1 and 3 μM arsenite strongly promoted anchorage-independent growth of HaCaT cells, no promotion of anchorage-independent growth was observed in HaCaT cells treated with 10 μM arsenite (Supplementary Figure S2 online). These results indicate that 1 and 3 μM arsenite are suitable concentrations for examination of arsenite-mediated cancer-promoting activity in nontumorigenic HaCaT cells.

Arsenite-mediated activation of PlGF expression and secretion

We then focused on PlGF as a regulatory factor for arsenite-mediated cancer promotion. Constitutive expression level of *PlGF* mRNA in A431 cells was more than 3-fold higher than that in HaCaT cells (Figure 2a). Expression level of *PlGF* mRNA in HaCaT cells was increased by treatment with 1 and 3 μM arsenite for 48 h (Figure 2a). Expression level of *PlGF* mRNA in HaCaT cells was about 4.0-fold increased at 3 h after 3 μM arsenite stimulation (Figure 2b). More than 2.6-fold increased expression of *PlGF* mRNA in HaCaT cells was still maintained at 48 h after 3 μM arsenite stimulation (Figure 2b). Expression levels of PlGF protein in HaCaT cells were also increased by treatment with 1 and 3 μM arsenite for 48 h (Figure 2c). We next examined the expression levels of PlGF protein in culture medium of HaCaT and A431 cells, because PlGF has been reported to be extracellularly secreted and to bind to vascular endothelial growth factor receptor 1 (VEGFR1) on the cellular surface (Fischer *et al.*, 2008). Constitutively secreted PlGF protein level from A431 cells cultured for 24 h was 8.7-fold higher than that from HaCaT cells (Figure 2d), and levels of PlGF protein secreted from cultured HaCaT cells treated with 1 and 3 μM arsenite for 24 h were 3.2- and 7.0-fold higher, respectively, than those from

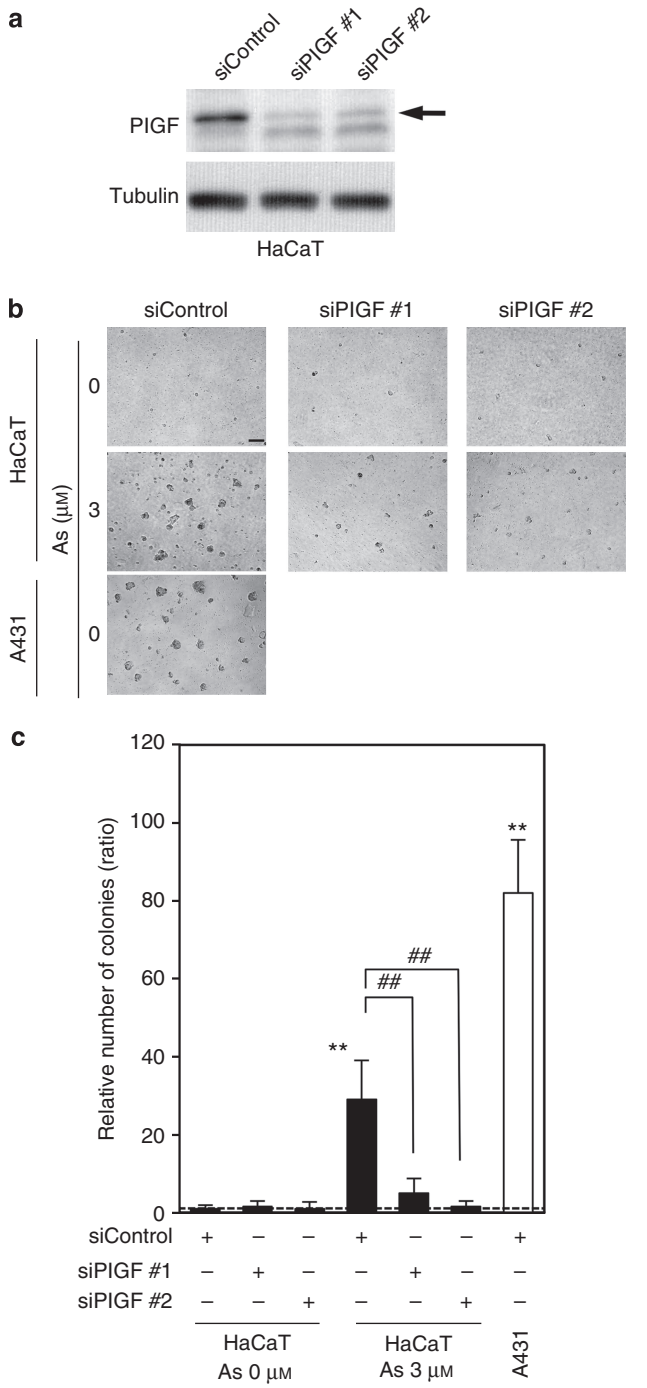

Figure 3. Inhibited anchorage-independent growth of placental growth factor (PlGF)-depleted HaCaT cells. (**a**) Expression levels of PlGF protein evaluated by immunoblotting in HaCaT cells, transfected with 25 nM of control small interfering RNA (siRNA; siControl) or siRNA for PlGF depletion (siPlGF #1 and #2) are presented. Amounts of tubulin are also presented as an internal control. (**b**) Colonies derived from siRNA-transfected HaCaT cells untreated or treated with 3 μM arsenite and from untreated A431 cells are presented. Bar = 100 μm. (**c**) Number of colonies per well derived from siRNA-transfected HaCaT cells (black bars) untreated or treated with 3 μM arsenite and from untreated A431 cells (a white bar) are presented as ratios (mean ± SD; $n = 3$) relative to untreated HaCaT cells with siControl transfection. Significantly different from untreated HaCaT cells with siControl transfection (**$P < 0.01$) and from 3 μM arsenite-treated HaCaT cells with siControl transfection ($^{\#\#}P < 0.01$) by Student's *t*-test.

untreated HaCaT cells (Figure 2d). The same results were obtained for levels of PlGF protein secreted from HaCaT cells cultured for 48 h (Figure 2d). These results indicated that 1 and 3 μM arsenite enhances the levels of PlGF expression in, and secretion from, HaCaT cells. In addition, expression levels of *PlGF* mRNA and protein in primary normal human epithelial keratinocyte cells were also increased by treatment with 1 and 3 μM arsenite for 48 h (Supplementary Figure S3 online), suggesting that PlGF expression is activated by arsenic exposure in primary human keratinocyte cells, as well as a human keratinocyte cell line.

Inhibition of arsenite-mediated anchorage-independent growth of PlGF-depleted HaCaT cells

We next examined whether increased PlGF is involved in anchorage-independent growth in HaCaT cells. PlGF was depleted from HaCaT cells by small interfering RNA transfection (Figure 3a), and anchorage-independent growth of cells treated with arsenite was examined (Figure 3b and c). Arsenite-mediated anchorage-independent growth of PlGF-depleted HaCaT cells was suppressed by 82.8–94.5% (Figure 3c). These results suggest that PlGF might be a regulator for arsenite-mediated anchorage-independent growth of HaCaT cells.

Arsenite-mediated activation of VEGFR1

As previous studies revealed that PlGF binds to VEGFR1 and increases phosphorylated levels of VEGFR1 (Autiero *et al.*, 2003), we next examined the influence of arsenite on phosphorylated levels of VEGFR1 in HaCaT cells (Figure 4a–d). Constitutive level of VEGFR1 phosphorylation in HaCaT cells was undetectably low, despite constitutively phosphorylated VEGFR1 in A431 cells (Figure 4a). Arsenite (1 and 3 μM) increased the levels of VEGFR1 phosphorylation in HaCaT cells, whereas there were no effects of arsenite on expression levels of *VEGFR1* mRNA and protein (Figure 4a and b). In addition, arsenite had no effect on phosphorylation and/or expression levels of VEGFR2 and *VEGFA* in HaCaT cells (Figure 4c and d).

Arsenite-mediated activation of MEK/ERK signaling

We next examined the effect of arsenite on the activation of signal transduction molecules potentially sited downstream of PlGF/VEGFR1 signaling. Previous studies showed that signaling of activated VEGFR1 was transduced to intercellular signal transduction including MEK/ERK, phosphoinositide 3-kinase (PI3K)/AKT, and focal adhesion kinase (FAK) signaling pathways, which are associated with cancer promotion (Gille *et al.*, 2000; Matsumoto *et al.*, 2002; Selvaraj *et al.*, 2003). Arsenite (1 and 3 μM) activated the phosphorylation levels of MEK/ERK but not PI3K/AKT and FAK signaling in HaCaT cells (Figure 4e). Moreover, arsenite-mediated phosphorylation of VEGFR1/MEK/ERK was suppressed in PlGF-depleted HaCaT cells (Figure 4f).

Arsenite-mediated degradation of MTF-1 in a proteasome-dependent manner

As our results provided evidence that arsenite-mediated increase of PlGF could be an important trigger for promotion

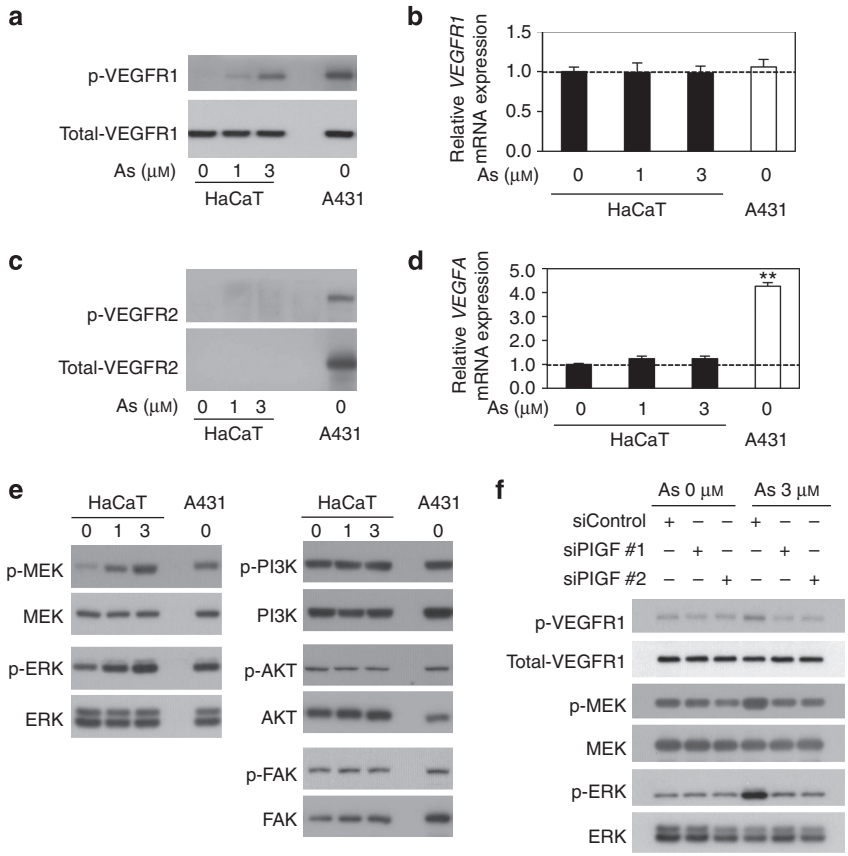

Figure 4. Arsenite-induced modification of placental growth factor (PlGF)-related signaling activities. (**a**) Phosphorylation and protein levels of vascular endothelial growth factor receptor1 (VEGFR1) in HaCaT cells untreated or treated with 1 and 3 μM arsenite for 48 h and in untreated A431 cells are presented. (**b**) Expression levels of *VEGFR1* messenger RNA (mRNA) in HaCaT cells untreated or treated with 1 and 3 μM arsenite for 48 h (black bars) and in untreated A431 cells (a white bar) are presented. (**c**) Phosphorylation and expression levels of VEGFR2 protein in HaCaT cells untreated or treated with 1 and 3 μM arsenite for 48 h and in untreated A431 cells are presented. (**d**) Expression levels of *VEGFA* mRNA in HaCaT cells untreated or treated with 1 and 3 μM arsenite for 48 h (black bars) and in untreated A431 cells (a white bar) are presented. The results in **b** and **d** are presented as ratios (mean ± SD; $n = 3$) of *VEGFR1* and *VEGFA* mRNA evaluated by real-time PCR relative to untreated HaCaT cells. Significantly different (**$P < 0.01$) from untreated HaCaT cells by Student's *t*-test. (**e**) Phosphorylation and expression levels of MEK, ERK, PI3K, AKT, and FAK proteins in HaCaT cells untreated or treated with 1 and 3 μM arsenite for 48 h and in untreated A431 cells are presented. (**f**) Phosphorylation and expression levels of VEGFR1, MEK, and ERK proteins in HaCaT cells, transfected with 25 nM of control small interfering RNA (siRNA; siControl) or siRNA for PlGF depletion (siPlGF #1 and #2), untreated or treated with 3 μM arsenite for 48 h are presented.

of anchorage-independent growth, we investigated the molecular mechanism for arsenite-mediated induction of PlGF expression in HaCaT cells. Previous studies showed that several transcription factors, including MTF-1, regulate promoter activity of the *PlGF* gene (Nishimoto *et al.*, 2009). As MTF-1 is a highly conserved protein in the transcriptional response to exposure to various elements including arsenite (Lichtlen and Schaffner, 2001; He and Ma, 2009), we examined the effect of arsenite on MTF-1 protein expression in HaCaT cells. Interestingly, the amount of MTF-1 protein in HaCaT cells treated with 3 μM arsenite was significantly decreased compared with that in untreated HaCaT cells (Figure 5a). A proteasome inhibitor (MG-132), but not a lysosome inhibitor (chloroquine), suppressed the arsenite-mediated decrease of MTF-1 protein (Figure 5a). We then performed chromatin immunoprecipitation assays to examine the amount of MTF-1 binding to the *PlGF* promoter in arsenite-treated and arsenite-untreated HaCaT cells. According to a previous study (Nishimoto *et al.*, 2009), a pair of primers to amplify the 3x metal responsive element region of the *PlGF* promoter was used for PCR and real-time PCR (Figure 5b and c). Relative occupancy of MTF-1 on the *PlGF* promoter in HaCaT cells treated with 3 μM arsenite was less than half of that in untreated control HaCaT cells (Figure 5c). As cadmium-mediated degradation of Mtf-1 in a murine hepatoma cell line (hepa1c1c7) was shown in a previous study (Bi *et al.*, 2006), our results suggest that arsenite induces proteasome-dependent degradation of MTF-1, resulting in a decreased amount of MTF-1 binding to the metal-responsive element region of the *PlGF* promoter in HaCaT cells.

Increased PlGF expression in, and secretion from, MTF1-depleted HaCaT cells

To address the biological effect of decreased expression of MTF-1 on PlGF, we examined the levels of *PlGF* expression in, and secretion from, MTF-1-depleted HaCaT cells by small

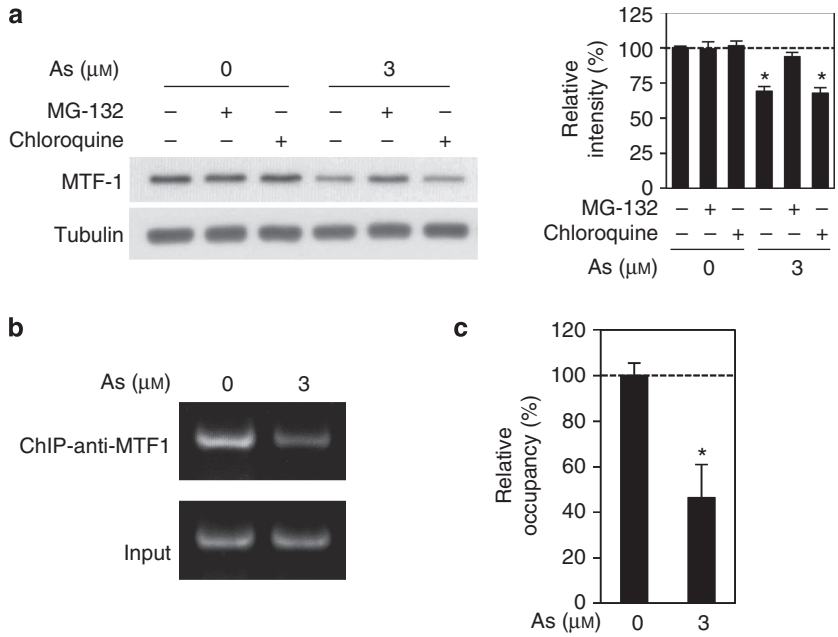

Figure 5. Arsenite-mediated degradation of MTF-1 protein and inhibition of binding to the *PlGF* promoter of metal-regulatory transcription factor-1 (MTF-1). (**a**) Expression levels of MTF-1 protein in HaCaT cells untreated or treated with 3 μM arsenite in the presence or absence of MG-132 (a proteasome inhibitor) or chloroquine (a lysosome inhibitor) for 48 h are presented. After treatment with MG-132 or chloroquine for 3 h for the protease inhibition, the cells were treated with arsenite for 24 h. Expression levels of TUBULIN protein are also presented as an internal control. Intensities of bands are presented as percentages (mean ± SD; $n = 3$) relative to untreated HaCaT cells. (**b** and **c**) HaCaT cells untreated or treated with 3 μM arsenite were cultured for 48 h before a chromatin immunoprecipitation assay with anti-MTF-1 antibody. Amplified bands evaluated by normal PCR are presented (**b**). Relative occupancy of MTF-1 from precipitated DNA in arsenite-treated HaCaT cells evaluated by real-time PCR is presented as percentage (mean ± SD; $n = 3$) of that in untreated HaCaT cells (**c**). Significantly different ($^*P < 0.05$) from untreated HaCaT cells by Student's *t*-test.

interfering RNA transfection in the presence or absence of 3 μM arsenite (Figure 6). The level of *PlGF* mRNA expression in MTF-1-depleted (siMTF-1 #1 and #2) HaCaT cells was 15.4- to 16.3-fold higher than that in control (siControl) HaCaT cells (Figure 6b). The level of PlGF protein secreted from MTF-1-depleted HaCaT cells cultured for 48 h was 5.7- to 6.4-fold increased compared with that from control HaCaT cells (Figure 6c). These results suggest increased PlGF expression and secretion levels by depletion of MTF-1 in HaCaT cells. *PlGF* mRNA expression level in MTF-1-depleted HaCaT cells treated with arsenite was further increased compared with that in control HaCaT cells treated with arsenite (Figure 6b). Levels of PlGF protein secreted from MTF-1-depleted HaCaT cells treated with arsenite were slightly increased but statistically insignificant compared with those from control HaCaT cells treated with arsenite (Figure 6c). Thus, our results showed that levels of PlGF expression in, and secretion from, MTF-1-depleted HaCaT cells treated with arsenite were 28.4- to 30.1-fold and 7.5- to 7.6-fold higher, respectively, than levels of expression in, and secretion from, control HaCaT cells that were not treated with arsenite (Figure 6b and c). Previous studies showed that MTF-1 enhanced the transcription of *metallothionein IIA (MT2A)* and *metallothionein IG (MT1G)* genes through metal-responsive elements on their promoter regions (Brugnera *et al.*, 1994; Joshi *et al.*, 2005). Induction of arsenite-mediated *MT2A* expression was inhibited in MTF-1-depleted HaCaT cells in our study (Figure 6d). MTF-1 depletion inhibited not only arsenite-mediated enhancement of *MT1G* expression but also constitutive expression of *MT1G* in HaCaT cells (Figure 6e).

DISCUSSION

In this study, our fieldwork demonstrated increased urinary levels of PlGF in residents of cancer-prone areas in Bangladesh who drink arsenic-polluted well water. We also experimentally demonstrated a unique molecular mechanism by which arsenite-mediated degradation of MTF-1 may enhance PlGF expression in, and secretion from, nontumorigenic cutaneous cells, resulting in an increase of transforming activity via activation of VEGFR1/MEK/ERK signaling (Figure 6f). Our results suggest that arsenite exposure from well drinking water enhances PlGF expression and secretion via MTF-1 degradation and promotes skin cancer.

A previous study showed increased levels of PlGF expression in SCC (Cheng *et al.*, 2010). Our fieldwork showed high levels of urinary PlGF in residents of cancer-prone areas, suggesting increased levels of PlGF in people at high risk for SCC. We then experimentally showed that 1 and 3 μM arsenite increased anchorage-independent growth with enhanced PlGF expression in, and secretion from, nontumorigenic HaCaT cells. Arsenite-mediated activation of anchorage-independent growth was more than 80% repressed by depletion of PlGF in HaCaT cells. Our results also suggest that urinary levels of PlGF might be useful for prediction of the

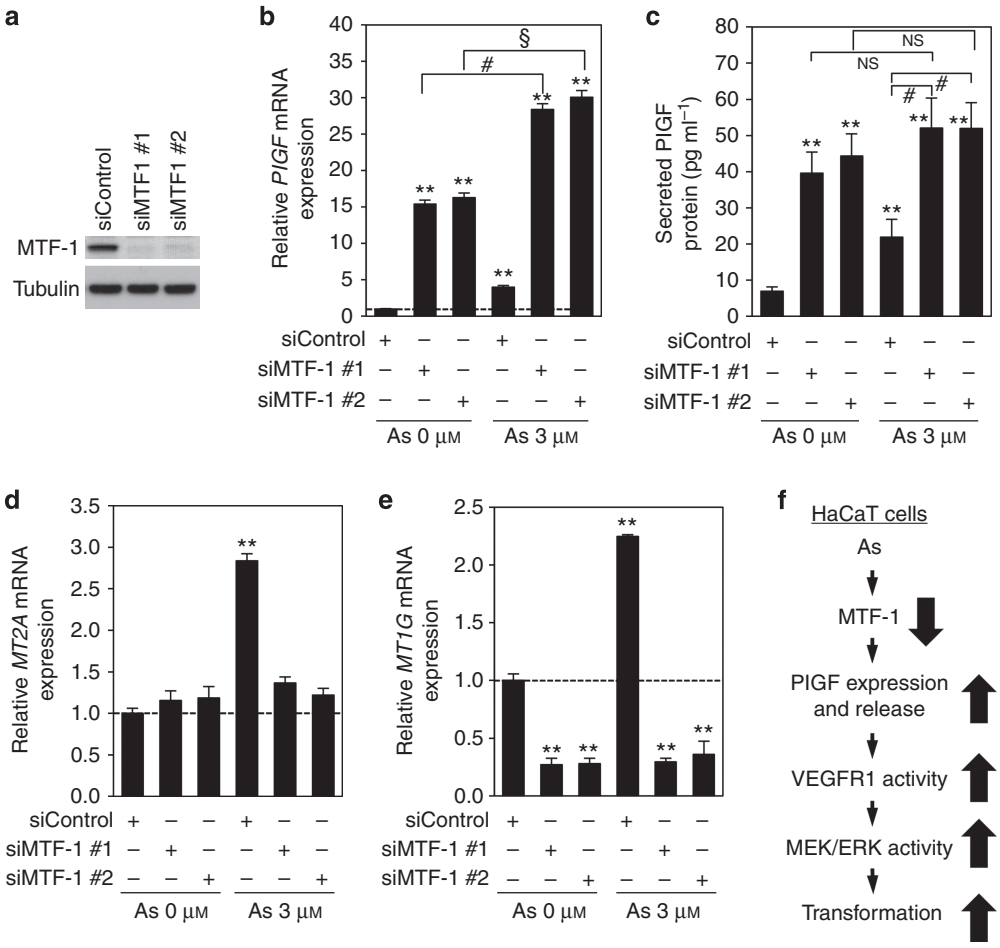

Figure 6. Expression levels of placental growth factor (PlGF), MT2A, and MT1G in metal-regulatory transcription factor-1 (MTF-1)-depleted HaCaT cells treated with arsenite. (**a**) Expression levels of MTF-1 protein evaluated by immunoblotting in HaCaT cells, transfected with 25 nM control small interfering RNA (siRNA; siControl) or siRNA for MTF-1 depletion (siMTF-1 #1 and #2), are presented. Amounts of tubulin are also presented as an internal control. (**b**) Expression levels of *PlGF* messenger RNA (mRNA) evaluated by real-time PCR in siRNA-transfected HaCaT cells untreated or treated with 3 μM arsenite are presented. The results are presented as ratios (mean ± SD; $n=3$) of *PlGF* mRNA relative to HaCaT cells untreated with arsenite. (**c**) Amounts of secreted PlGF protein evaluated by ELISA in culture medium of HaCaT cells, transfected with 25 nM of control siRNA (siControl) or siRNA for MTF-1 depletion (siMTF-1 #1 and #2), in the absence or presence of 3 μM arsenite are presented. Expression levels of *MT2A* (**d**) and *MT1G* (**e**) mRNAs evaluated by real-time PCR in HaCaT cells transfected with 25 nM control siRNA (siControl) or siRNA for MTF-1 depletion (siMTF1 #1 and #2) untreated or treated with 3 μM arsenite are presented. The results are presented as ratios (mean ± SD; $n=3$) of *MT2A* and *MT1G* mRNA relative to HaCaT cells transfected with 25 nM control siRNA (siControl). Significantly different from untreated HaCaT cells with siControl transfection (**$P<0.01$ in **b**–**e**), from untreated HaCaT cells with siMTF-1 #1 transfection ($^{\#}P<0.05$ in **b**) or with siMTF-1 #2 transfection ($^{\S}P<0.05$ in **b**) and from 3 μM arsenite-treated HaCaT cells with siControl transfection ($^{\#}P<0.05$ in **c**) by Student's *t*-test. NS, not significant. (**f**) Our proposal for the potential pathway of arsenite-mediated transformation in HaCaT cells is presented as a schema.

transformation of keratinocytes in patients with arsenicosis, because anchorage-independent growth is one of the important hallmarks of transformation from normal cells to cancer cells (Kato *et al.*, 2002). In addition, decreased PlGF level reduces the number of endothelial cells and neovascularization in PlGF knockout mice (Carmeliet *et al.*, 2001). Monoclonal anti-Flt1 antibody MF1 (anti-Flt1), which blocked binding of VEGF and PlGF to Flt1, inhibited growth and angiogenesis of tumors derived from A431 cells subcutaneously injected into nude mice (Luttun *et al.*, 2002). Therefore, PlGF may also be associated with angiogenesis in arsenite-induced skin cancer.

HaCaT cells have compound heterozygous mutations in each p53 allele that show decrease of protein activities (Lehman *et al.*, 1993). Wild-type p53 (WT-p53) is associated with apoptosis, and downregulation of p53 activity is an important step in the progression of cancer, including SCC (Brash, 2006). The role of WT-p53 in arsenite-induced anchorage-independent growth of HaCaT cells was analyzed by using transfection of a WT-p53 expression vector into HaCaT cells (Supplementary Figure S4 online). Ectopic expression of Halo-tag-fused WT-p53 was detected at 86 kDa (Supplementary Figure S4a online), and it increased the expression level of $p21^{Cip1/WAF1}$, a direct target of p53 and a tumor-suppressor gene (Abbas and Dutta, 2009; Supplementary Figure S4b online). Anchorage-independent growth was observed in HaCaT cells ectopically expressing WT-p53 and GFP for tracing transfected cells treated with 3 μM arsenite

(Supplementary Figure S4i and j online). Levels of anchorage-independent growth of WT-p53-transfected HaCaT cells treated with 3 μM arsenite were significantly lower than those in empty vector–transfected HaCaT cells treated with 3 μM arsenite (Supplementary Figure S4k online). These results suggest that HaCaT cells expressing WT-p53 are not only viable but also show an increased level of anchorage-independent growth under the condition of treatment with 3 μM arsenite.

We further analyzed the molecular mechanism of PlGF for arsenite-mediated cancer promotion in HaCaT cells. Since we found that arsenite increased VEGFR1 activity without affecting VEGFR1 expression, arsenite may have enhanced VEGFR1 activity by increasing PlGF secretion. Although PlGF does not directly bind to VEGFR2, PlGF affects phosphorylation and/or expression of VEGFA and VEGFR2 through intermolecular cross talk of activated VEGFR1 (Roy *et al.*, 2005). Therefore, we next examined whether arsenite affects phosphorylated and/or expression levels of VEGFR2 and VEGFA. The results showed that there was no expression of VEGFR2 and very limited effects of arsenite on phosphorylated and/or expression levels of VEGFR2 and VEGFA in HaCaT cells. Our results suggest that VEGFR1 activation through arsenite-mediated increase of PlGF expression and release enhanced activities of MEK/ERK but not those of PI3K/AKT and FAK (Figure 6f).

We finally analyzed the molecular mechanism of arsenite-mediated enhancement of PlGF expression. Previous studies showed that MTF-1 acted as a positive regulator for PlGF expression in human embryonic kidney HEK293 cells and human choriocarcinoma BeWo cells under a hypoxia condition (Cramer *et al.*, 2005; Nishimoto *et al.*, 2009). Therefore, we assumed that arsenite-mediated increase of MTF-1 would enhance PlGF expression in HaCaT cells. However, contrary results were obtained in this study. Arsenite induced proteasome-dependent degradation of MTF-1 protein, resulting in a decreased level of MTF-1 binding to the *PlGF* promoter and enhanced levels of PlGF expression in, and secretion from, HaCaT cells. These results suggest that the mechanism of MTF-1-mediated regulation of PlGF expression in HaCaT cells might be different from that in HEK293 and BeWo cells. On the other hand, expression of MT2A and MT1G, other target genes of MTF-1 (Brugnera *et al.*, 1994; Joshi *et al.*, 2005), was inhibited in MTF-1-depleted HaCaT cells. Thus, MTF-1-mediated positive regulation for *MT2A* and *MT1G* mRNA expression in HaCaT cells corresponds to the results of previous studies (Brugnera *et al.*, 1994; Joshi *et al.*, 2005). MTF-1 might exhibit different functions in different genes and cells and with different environmental stimulations. The reason why MTF-1 acts as a negative regulator for PlGF expression in HaCaT cells remains unknown. A recent study showed that the N-terminal domain of MTF-1, which includes a nuclear import and export signaling sequence and a zinc finger domain, inhibits its own transcriptional activity (He and Ma, 2009). The results of that study also suggested that the N-terminal domain of MTF-1 interacted with a repressor protein, resulting in the inhibition of transcription of its target genes (He and Ma, 2009). Moreover, levels of anchorage-independent growth were decreased in PlGF-depleted A431 cells, whereas levels of PlGF expression were increased in MTF-1-depleted A431 cells (Supplementary Figure S5 online). These results suggest that the same mechanism of increased levels of anchorage-independent growth through increase of *PlGF* expression mediated by MTF-1 downregulation is involved in both HaCaT and A431 cells.

In conclusion, results of our studies may have provided an insight into the role of PlGF in arsenite-mediated skin cancer *in vitro*. Further study should be carried out to determine whether PlGF is useful for the prevention of arsenite-mediated skin cancer.

MATERIALS AND METHODS

Measurements of arsenic in well water and measurements of urinary arsenic and PlGF

Samples of well drinking water ($n=15$) and human urine ($n=61$) were collected in Samta and Tangra, previously reported cancer-prone areas, Jessore in Bangladesh (Kurokawa *et al.*, 2001; Yokota *et al.*, 2002; Tareq *et al.*, 2003). Details are described in Supplementary Informationonline.

Reagents

See Supplementary Information online.

Cell culture

HaCaT and A431 cells were supplied from Cell Line Service (Eppelheim, Germany) and RIKEN BioResource Center (Tsukuba, Japan), respectively. Details are described in Supplementary Information online.

Colony-formation assay

Cells (2×10^4) were added to 1 ml of 1.5% methylcellulose/DMEM and treated or not treated with arsenite in 24-well culture plates with Ultra-low attachment surfaces (Corning, NY). Details are described in Supplementary Informationonline.

Real-time PCR

Total RNA was prepared from culture cells using a High Pure RNA Purification Kit (Roche, Basel, Switzerland) according to the manufacturers' protocol. Details are described in Supplementary Information online.

Immunoblotting

See Supplementary Information online.

RNA interference

Small interfering RNA–mediated depletion (knockdown) of *PlGF* and *MTF-1* was performed with nucleotide synthetic duplexes (Invitrogen, MA). Details are described in Supplementary Information online.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay was performed by using a Simple ChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology, MA). Details are described in Supplementary Information online.

Mice and exposure to arsenite and immunohistochemistry

Hairless homozygous mice (*Hr/Hr*) at 4 weeks of age were used for exposure experiments ($n=5$ in each concentration of arsenite). Details are described in Supplementary Information online.

Transfection of expression vectors

See Supplementary Information online.

Statistics

The SPSS (version 18) software package (IBM, NY) was used for statistical analyses by Student's *t*-test and the Mann–Whitney *U*-test.

Ethics statement

Sampling in Bangladesh was ethically approved by the Ethics Committee (approval no. 2013-0070-3 in Nagoya University and 250007 in Chubu University), Japan. The experiments using recombinant DNA were approved by the Recombination DNA Advisory Committee (approval no. 12-06 in Chubu University and 13-35 in Nagoya University) and the Animal Care and Use Committee (approval no. 2510052 in Chubu University and 26317 in Nagoya University), Japan.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Abbas T, Dutta A (2009) p21 in cancer: intricate networks and multiple activities. *Nat Rev Cancer* 9:400–14

Autiero M, Waltenberger J, Communi D *et al.* (2003) Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat Med* 9:936–43

Bi Y, Lin GX, Millecchia L *et al.* (2006) Superinduction of metallothionein I by inhibition of protein synthesis: role of a labile repressor in MTF-1 mediated gene transcription. *J Biochem Mol Toxicol* 20:57–68

Boukamp P, Petrussevska RT, Breitkreutz D *et al.* (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol* 106:761–71

Brash DE (2006) Roles of the transcription factor p53 in keratinocyte carcinomas. *Br J Dermatol* 154(Suppl 1):8–10

Brugnera E, Georgiev O, Radtke F *et al.* (1994) Cloning, chromosomal mapping and characterization of the human metal-regulatory transcription factor MTF-1. *Nucleic Acids Res* 22:3167–73

Carmeliet P, Moons L, Luttun A *et al.* (2001) Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med* 7:575–83

Cheng SJ, Lee JJ, Kok SH *et al.* (2010) Expression of placenta growth factor: an independent factor for prediction of progression and prognosis of oral cancer. *Head Neck* 32:1363–9

Chowdhury UK, Biswas BK, Chowdhury TR *et al.* (2000) Groundwater arsenic contamination in Bangladesh and West Bengal, India. *Environ Health Perspect* 108:393–7

Cramer M, Nagy I, Murphy BJ *et al.* (2005) NF-kappaB contributes to transcription of placenta growth factor and interacts with metal responsive transcription factor-1 in hypoxic human cells. *Biol Chem* 386:865–72

Fischer C, Jonckx B, Mazzone M *et al.* (2007) Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. *Cell* 131:463–75

Fischer C, Mazzone M, Jonckx B *et al.* (2008) FLT1 and its ligands VEGFB and PlGF: drug targets for anti-angiogenic therapy? *Nat Rev Cancer* 8:942–56

Gault AG, Rowland HA, Charnock JM *et al.* (2008) Arsenic in hair and nails of individuals exposed to arsenic-rich groundwaters in Kandal province, Cambodia. *Sci Total Environ* 393:168–76

Gille H, Kowalski J, Yu L *et al.* (2000) A repressor sequence in the juxtamembrane domain of Flt-1 (VEGFR-1) constitutively inhibits vascular endothelial growth factor-dependent phosphatidylinositol 3′-kinase activation and endothelial cell migration. *EMBO J* 19:4064–73

Han W, Ming M, He TC *et al.* (2010) Immunosuppressive cyclosporin A activates AKT in keratinocytes through PTEN suppression: implications in skin carcinogenesis. *J Biol Chem* 285:11369–77

Hata A, Yamanaka K, Habib MA *et al.* (2012) Arsenic speciation analysis of urine samples from individuals living in an arsenic-contaminated area in Bangladesh. *Environ Health Prevent Med* 17:235–45

He X, Ma Q (2009) Induction of metallothionein I by arsenic via metal-activated transcription factor 1: critical role of C-terminal cysteine residues in arsenic sensing. *J Biol Chem* 284:12609–21

Hinwood AL, Sim MR, Jolley D *et al.* (2003) Hair and toenail arsenic concentrations of residents living in areas with high environmental arsenic concentrations. *Environ Health Perspect* 111:187–93

IARC (1980) Some Metals and Metallic Compounds. *Monogr Eval Carcinog Risk Chem Hum* 23:37–141

IARC (2004) Some drinking-water disinfectants and contaminants, including arsenic. *IARC Monogr Eval Carcinog Risks Hum* 84:1–477

Joshi B, Ordonez-Ercan D, Dasgupta P *et al.* (2005) Induction of human metallothionein 1G promoter by VEGF and heavy metals: differential involvement of E2F and metal transcription factors. *Oncogene* 24: 2204–17

Kato M, Onuma S, Kato Y *et al.* (2010) Toxic elements in well water from Malaysia. *Toxicol Environ Chem* 92:1609–12

Kato M, Takeda K, Kawamoto Y *et al.* (2002) Repair by Src kinase of function-impaired RET with multiple endocrine neoplasia type 2A mutation with substitutions of tyrosines in the COOH-terminal kinase domain for phenylalanine. *Cancer Res* 62:2414–22

Kumasaka MY, Yamanoshita O, Shimizu S *et al.* (2012) Enhanced carcinogenicity by coexposure to arsenic and iron and a novel remediation system for the elements in well drinking water. *Arch Toxicol* 87:439–47

Kurokawa M, Ogata K, Idemori M *et al.* (2001) Investigation of skin manifestations of arsenicism due to intake of arsenic-contaminated groundwater in residents of Samta, Jessore, Bangladesh. *Arch Dermatol* 137:102–3

Lansdown AB (1995) Physiological and toxicological changes in the skin resulting from the action and interaction of metal ions. *Crit Rev Toxicol* 25:397–462

Lassen U, Nielsen DL, Sorensen M *et al.* (2012) A phase I, dose-escalation study of TB-403, a monoclonal antibody directed against PlGF, in patients with advanced solid tumours. *Br J Cancer* 106:678–84

Lehman TA, Modali R, Boukamp P *et al.* (1993) p53 mutations in human immortalized epithelial cell lines. *Carcinogenesis* 14:833–9

Li C, Xu J, Li F *et al.* (2011) Unfolded protein response signaling and MAP kinase pathways underlie pathogenesis of arsenic-induced cutaneous inflammation. *Cancer Prevent Res* 4:2101–9

Lichtlen P, Schaffner W (2001) Putting its fingers on stressful situations: the heavy metal-regulatory transcription factor MTF-1. *Bioessays* 23:1010–7

Loges S, Schmidt T, Carmeliet P (2009) 'Antimyeloangiogenic' therapy for cancer by inhibiting PlGF. *Clin Cancer Res* 15:3648–53

Luttun A, Tjwa M, Moons L *et al.* (2002) Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med* 8:831–40

Martinsson-Niskanen T, Riisbro R, Larsson L *et al.* (2011) Monoclonal antibody TB-403: a first-in-human, Phase I, double-blind, dose escalation study directed against placental growth factor in healthy male subjects. *Clin Ther* 33:1142–9

Matsui M, Nishigori C, Toyokuni S *et al.* (1999) The role of oxidative DNA damage in human arsenic carcinogenesis: detection of 8-hydroxy-2'-deoxyguanosine in arsenic-related Bowen's disease. *J Invest Dermatol* 113:26–31

Matsumoto Y, Tanaka K, Hirata G *et al.* (2002) Possible involvement of the vascular endothelial growth factor-Flt-1-focal adhesion kinase pathway in chemotaxis and the cell proliferation of osteoclast precursor cells in arthritic joints. *J Immunol* 168:5824–31

National-Research-Council (1999) *Arsenic in Drinking Water*. National Academy Press: Washington, DC

Nishimoto F, Sakata M, Minekawa R *et al.* (2009) Metal transcription factor-1 is involved in hypoxia-dependent regulation of placenta growth factor in trophoblast-derived cells. *Endocrinology* 150:1801–8

Parr C, Watkins G, Boulton M *et al.* (2005) Placenta growth factor is over-expressed and has prognostic value in human breast cancer. *Eur J Cancer* 41:2819–27

Roy H, Bhardwaj S, Babu M *et al.* (2005) Adenovirus-mediated gene transfer of placental growth factor to perivascular tissue induces angiogenesis via upregulation of the expression of endogenous vascular endothelial growth factor-A. *Hum Gene Ther* 16:1422–8

Selvaraj SK, Giri RK, Perelman N *et al.* (2003) Mechanism of monocyte activation and expression of proinflammatory cytochemokines by placenta growth factor. *Blood* 102:1515–24

Smith AH, Hopenhayn-Rich C, Bates MN *et al.* (1992) Cancer risks from arsenic in drinking water. *Environ Health Perspect* 97:259–67

Tareq SM, Safiullah S, Anawar HM *et al.* (2003) Arsenic pollution in groundwater: a self-organizing complex geochemical process in the deltaic sedimentary environment, Bangladesh. *Sci Total Environ* 313:213–26

Thang ND, Yajima I, Kumasaka MY *et al.* (2011) Barium promotes anchorage-independent growth and invasion of human HaCaT keratinocytes via activation of c-SRC kinase. *PLoS One* 6:e25636

Yajima I, Uemura N, Nizam S *et al.* (2012) Barium inhibits arsenic-mediated apoptotic cell death in human squamous cell carcinoma cells. *Arch Toxicol* 86:961–73

Yancy SL, Shelden EA, Gilmont RR *et al.* (2005) Sodium arsenite exposure alters cell migration, focal adhesion localization and decreases tyrosine phosphorylation of focal adhesion kinase in H9C2 myoblasts. *Toxicol Sci* 84:278–86

Yokota H, Tanabe K, Sezaki M *et al.* (2002) Arsenic contamination in groundwater of Samta, Bangladesh. *Water Sci Technol* 46:375–80